IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of			Date: March 6, 2007
BURANDA ET AL			Our File No. 7201102
Serial No.	:	09/985,873	Examiner Ann Y. Lam
Filed	:	11/06/2001	Group Art Unit 1641
For	:	Fluorescence and FRET Based Assays for Biomolecules on Beads	

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

DECLARATION UNDER § 1.131

We declare as follows:

1. We are named inventors of U.S. Patent Application Serial No. 09/985,873 ("the '873 application"), filed November 6, 2001, and entitled "Fluorescence and FRET Based Assays for Biomolecules on Beads" which claims the benefit of U.S. Provisional Application 60/246,564 ("the '564 application"), entitled "Bead-based Assay for Epitope Tags and Porous Affinity Sensor with Fluorescence Detection," filed Nov. 8, 2000.

2. We conceived our invention as presently claimed in this country earlier than January 4, 2000, and at least as early as June, 1999.

3. From at least as early as June 1999 until November 8, 2000, the filing date of the '564 application, we diligently worked toward perfecting the filing of the parent application. Specifically, various experiments were conducted and manuscripts and presentations were prepared.

4. Unfortunately, the lab notebooks correlating to the work that was performed were destroyed by a flood in Mr. Buranda's office that took place on December 24, 2005 and January 1, 2007. Accordingly, we are submitting, as evidence, those items we have that survived the floods.

5. Exhibit 1 is two screen shots showing photographs of the presently claimed invention. Specifically, these photographs show the results of antiFLAG antibody binding to FLAG peptides on beads in a microfluidic channel. These photographs are of experiments performed by Mr. Buranda or under Mr. Buranda's direction in his laboratory on July 22, 1999. As shown in the screen shot, the files were created on July 22, 1999.

6. Exhibit 2 is a page that was prepared in preparation for a report. The page is undated, but is believed to have been prepared in late 1999 or some time in 2000. As shown, the upper right hand quadrant depicts and describes one of the photographs provided in Exhibit 1. The title of the page is FRET based detection of Analytes in Microfluidic Channels Using Flow Cytometry-Calibrated Streptavidin Coated Beads.

7. Exhibit 3 is three pages of data that was generated by experiments performed by Mr. Buranda on September 27, 1999, as shown in the center typed portions of the pages.

8. Exhibit 4 is a coverletter and abstract mailed by Mr. Buranda on or around December 15, 1999 for inclusion in the Biosensors 2000 Conference which was held May 24 - May 26 in San Diego, California. As shown, the abstract discusses FRET based detection of analytes using flow cytometric methods. The abstract was originally submitted online and a confirmation copy including the attached coverletter was sent by mail.

9. Exhibit 5 is a progress report that was prepared Mr. Lopez for the grant that originally funded the work. The progress report described work that was performed between

June 1, 1999 and May 31, 2000. As shown, page 2, bullet point 7 of the report states "Development of FRET based assays in microfluidic channels using flow cytometry calibrated beads as well defined platforms for molecular assembly and high surface area detection. Demonstrations included analysis of FLAG peptides in serum."

10. Exhibit 6 is two pages from an Invention Disclosure submitted by Mr. Lopez and Mr. Buranda in connection with the '873 application. The invention disclosure is dated March 7, 2001. However, the second page states that the invention was first conceived in June 1999. The page further states that evidence of conception can be shown in photographs and a manuscript. These photographs referred to are believed to be the photographs submitted in Exhibit 1.

11. Exhibit 7 is a manuscript that was prepared by Mr. Buranda and Mr. Lopez. The manuscript is believed to be a version of the manuscript that is referred to in the invention disclosure of Exhibit 6. On page 4, last sentence, the manuscript states, "Simultaneous detection of a diverse group of analytes can be achieved by packing, discrete segments of receptor bearing beads in a single affinity micro-column (work in progress)." The manuscript discusses the various other aspects of the present invention as currently claimed throughout. Furthermore, Fig. 3B in the manuscript clearly shows one of the photographs from Exhibit 1.

12. All acts set forth herein and/or relied upon for the purpose of establishing invention prior to January 4, 2000 were carried out in the United States.

13. We declare that all statements made herein of our knowledge are true and all statements made on information and belief are believed to be true. These statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code. We understand that

such willful false statements may jeopardize the validity of the application or any patent issuing there from.

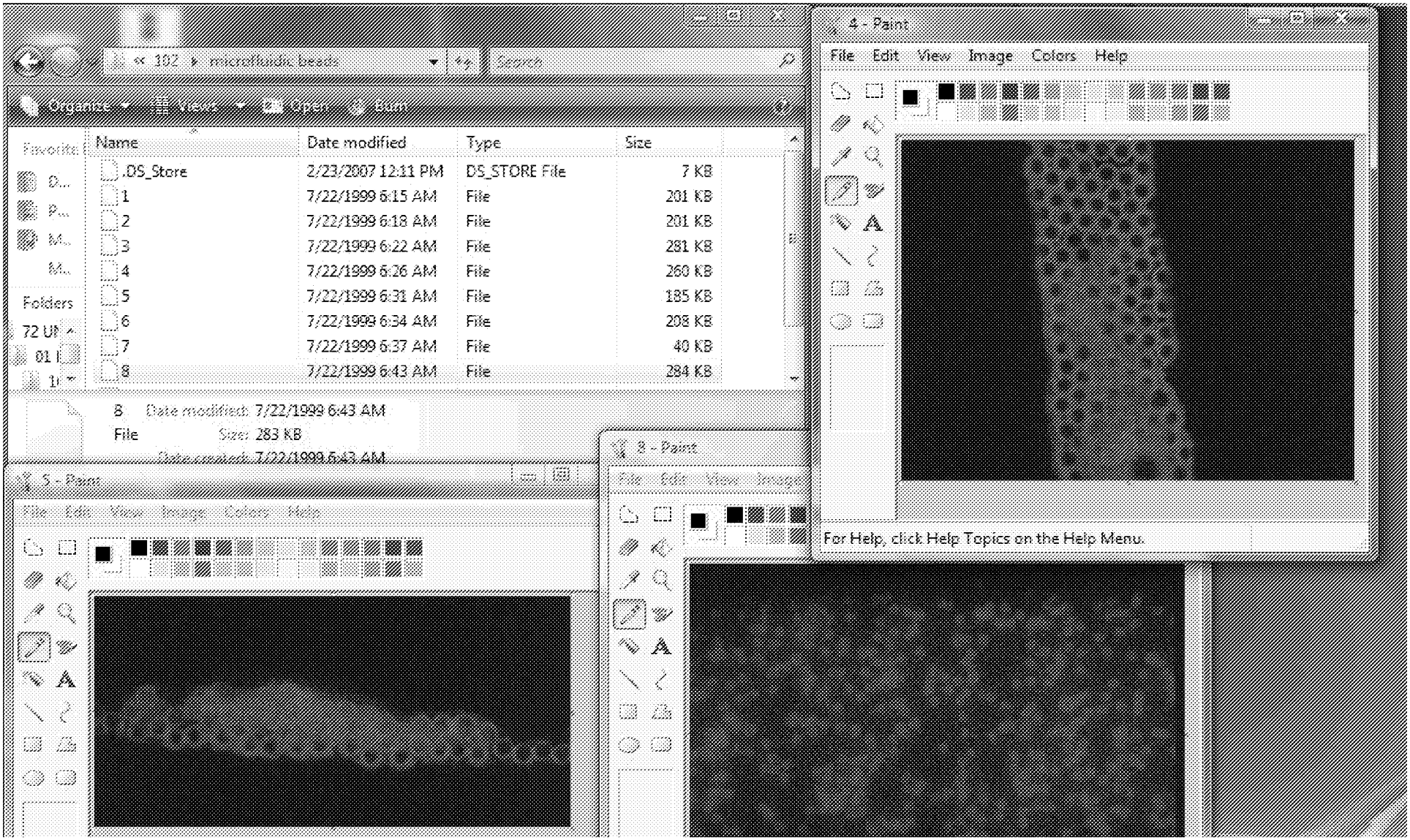
Date: 3/6/07

Tione Buranda

Date: 3/6/07

Gabriel Lopez

Exhibit 1


« 102 ▸ microfluidic beads
Search
Organize
Views
Open
Burn
Favorites
Folders
Name
Date modified
Type
Size
.DS_Store 2/23/2007 12:11 PM DS_STORE File 7 KB
1 7/22/1999 6:15 AM File 201 KB
2 7/22/1999 6:18 AM File 201 KB
3 7/22/1999 6:22 AM File 281 KB
4 7/22/1999 6:26 AM File 260 KB
5 7/22/1999 6:31 AM File 185 KB
6 7/22/1999 6:34 AM File 208 KB
7 7/22/1999 6:37 AM File 40 KB
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File Size: 283 KB
4 - Paint
File Edit View Image Colors Help
For Help, click Help Topics on the Help Menu.
5 - Paint
8 - Paint

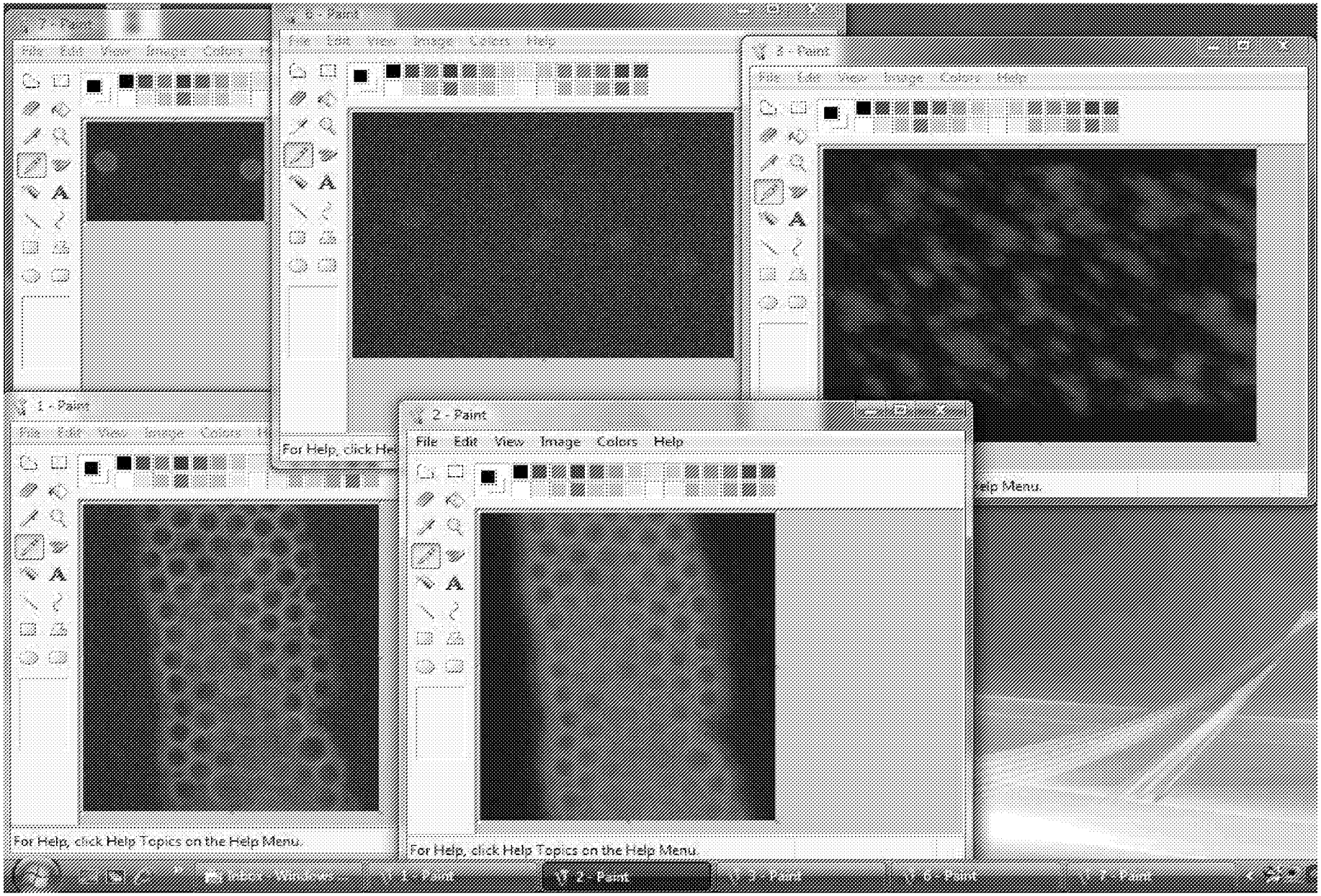
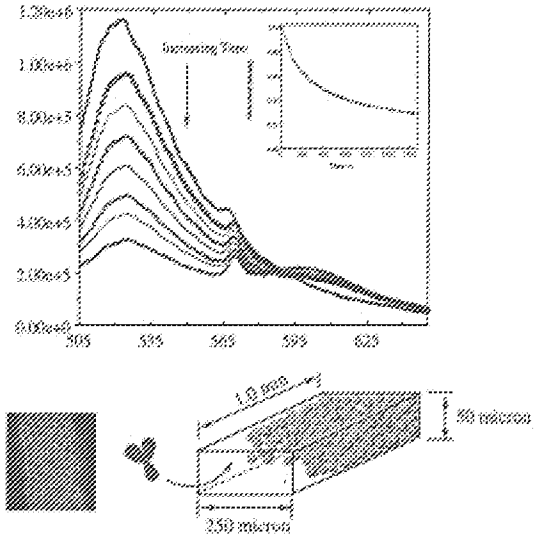

3 - Paint
File Edit View Image Colors Help
For Help, click Help Topics on the Help Menu.
1 - Paint
2 - Paint
For Help, click Help Topics on the Help Menu.

Exhibit 2

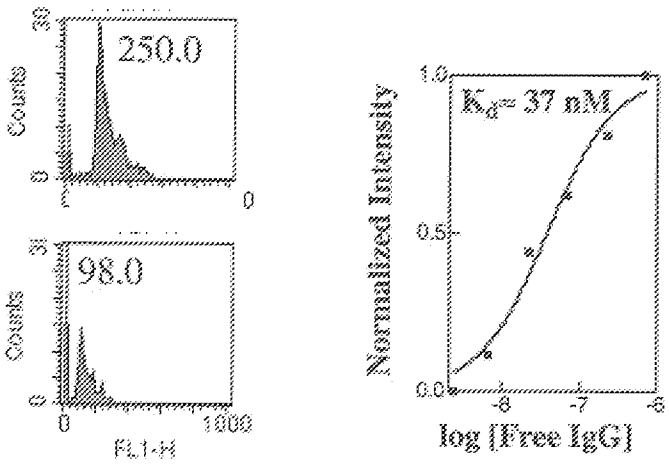
FRET based detection of Analytes in Microfluidic Channels Using Flow Cytometry-Calibrated Streptavidin Coated Beads.

- **The combination of flow cytometry and microfluidic channels enables the use of well calibrated surfaces to detect trace analytes.**
- **FRET assay facilitates distinction of free from bound IgG in microchannel**
- **High sensitivity allows up to attomole sensitivity**
- **Kinetic and Equilibrium study of analyte binding on a microscale**

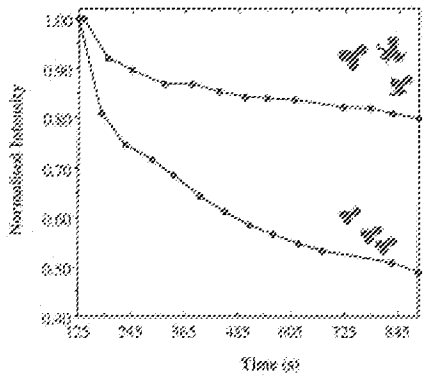
Kinetic Resolution of AntiFLAG Antibody Binding to FLAG Peptides on Beads in a Microfluidic Channel



Flow Cytometry histograms indicating FRETon beads bearing ≈ 6.6×10^{-14} moles of FLAG peptide before and after addition of ≈ 1.4×10^{-11} moles of anti-FLAG antibodies. Graph shows FRET based determination of IgG affinity to FLAG peptides on beads.



FRET Based Detection of FLAG Peptide in Whole Blood Serum via Competitive Assay with anti-FLAG Antibody in Microfluidic Channel.

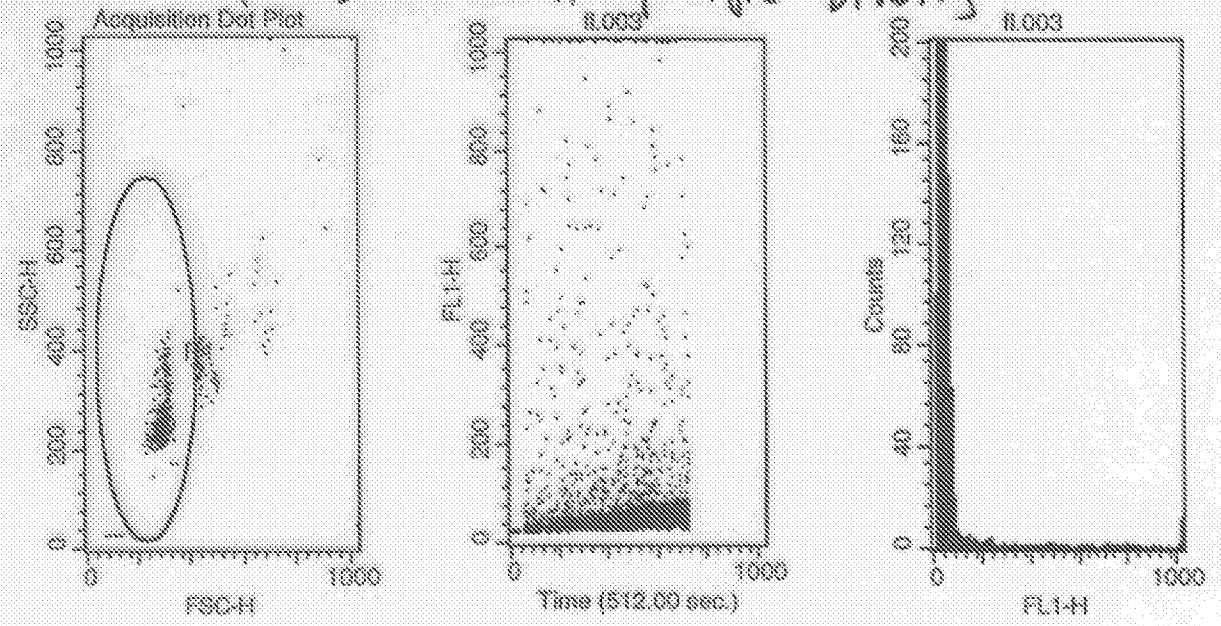


b. soluble pep. mixed with IgG: **less FRET**

a. Antibody alone flows into channel: **>50% FRET**

Exhibit 3

Binding of IgG to Flag pep without Ca2+ some non specific binding

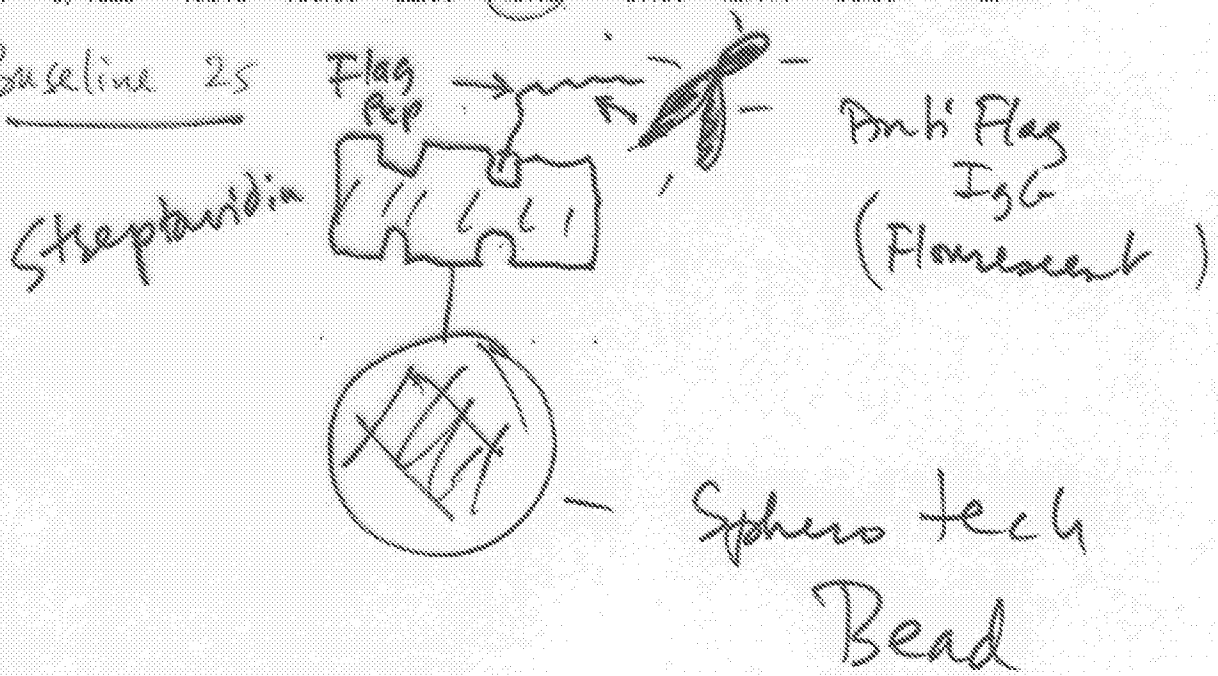


Histogram Statistics

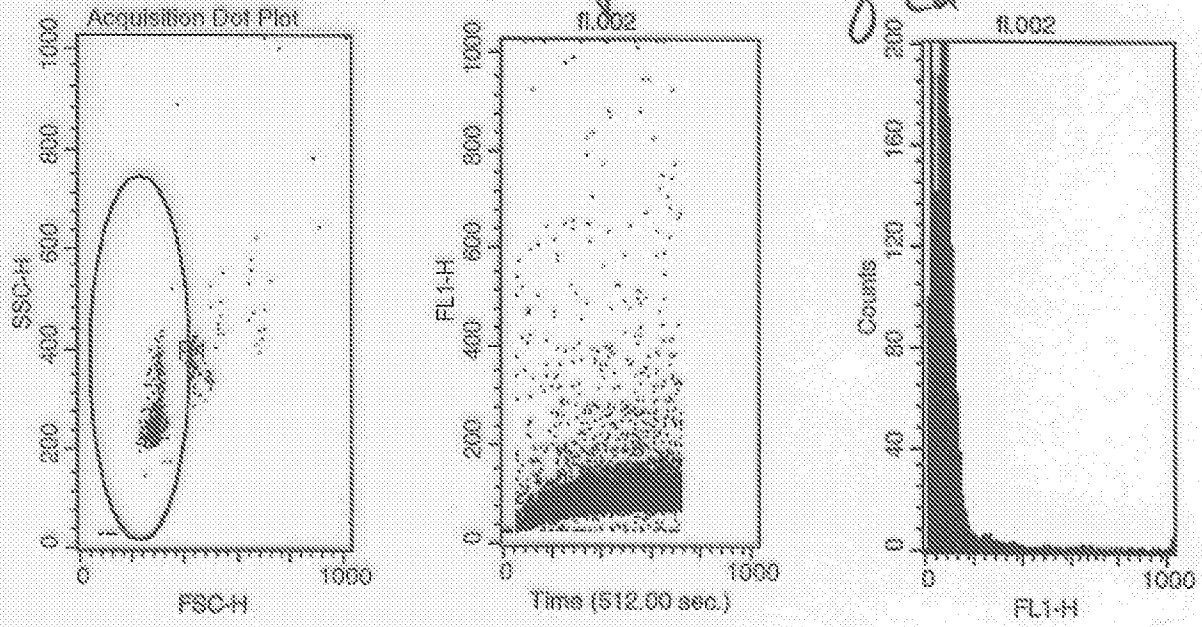
File: fl.003	Log Data Units: Linear Values
Sample ID:	Patient ID:
Patient Name:	Case Number:
Tube:	Panel:
Acquisition Date: 27-Sep-99	Gate: G1
Gated Events: 19346	Total Events: 33320
X Parameter: FL1-H (Linear)	

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	19346	100.00	58.06	43.12	37.55	127.41	36.00	27

Baseline 25



Binding of Flourescent Anti Flag IgG to pep in the presence of Ca^{2+}



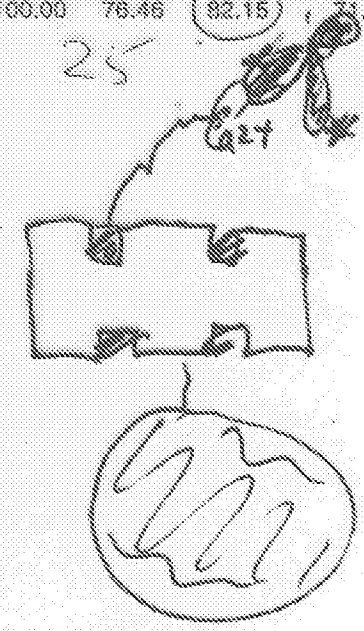
Histogram Statistics

File: fl.002
Sample ID:
Patient Name:
Tube:
Acquisition Date: 27-Sep-99
Gated Events: 17143
X Parameter: FL1-H (Linear)

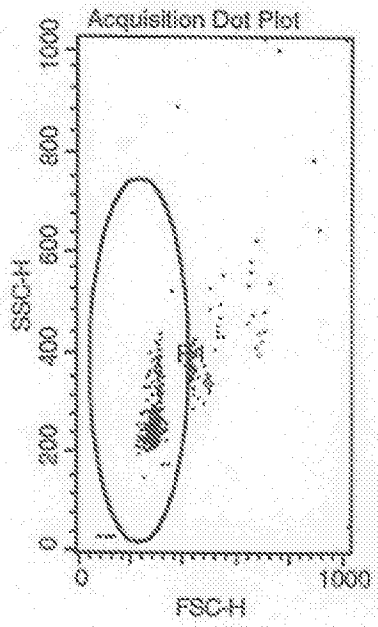
Log Data Units: Linear Values
Patient ID:
Case Number:
Panel:
Gate: G1
Total Events: 22429

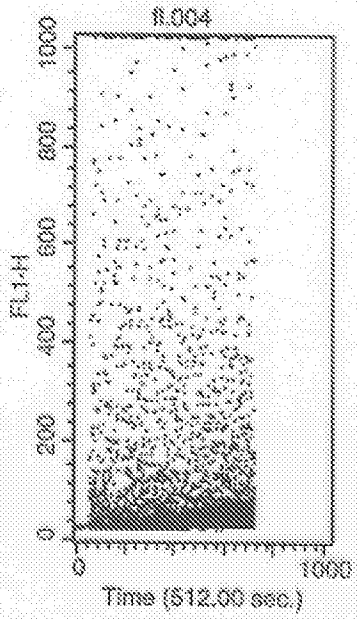
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	17143	100.00	76.46	82.15	71.70	73.30	76.00	25

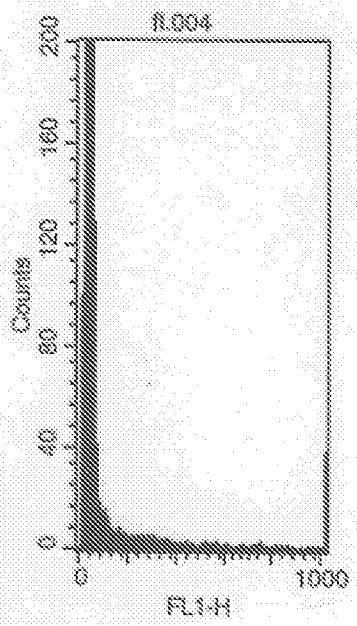
Base line 25



Non Specific binding of IgG to bead







Histogram Statistics

File: fl.004
Sample ID:
Patient Name:
Tube:
Acquisition Date: 27-Sep-99
Gated Events: 89176
X Parameter: FL1-H (Linear)

Log Data Units: Linear Values
Patient ID:
Case Number:
Panel:
Gate: G1
Total Events: 100740

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	89176	100.00	88.52	34.11	30.61	133.61	28.00	27

Base line 25

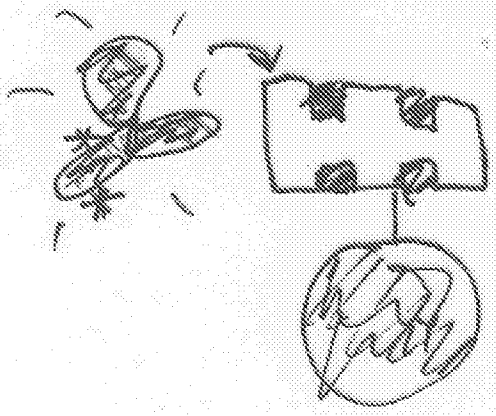


Exhibit 4

December 15, 1999

Liz Reed
Biosensors 2000 Conference Secretariat
Elsevier Science
The Boulervard
Langford Lane
Kidlington
Oxford, OX5 1GB
UK

Dear Ms. Reed:

Please find enclosed two original copies of our abstract "Reagentless and Regenerable Sensors on Beads" which was originally submitted at the conference website for your consideration for the Biosensors 2000 conference.

Sincerely,

Tione Buranda, Ph.D.

Reagentless and Regenerable Sensors on Beads

Tione Buranda,[1,2*] Toban Robledo,[2] Gabriel P. Lopez,[2] and Larry A. Sklar.[1,3]
[1]Cytometry Laboratory, Cancer Center and Department of Pathology, University of New Mexico School of Medicine, and [2]Department of Chemical and Nuclear Engineering, University of New Mexico, Albuquerque, NM 87131

We have taken a mechanistic approach to the development of a real-time, regenerable, multi-analyte sensors on beads. The detection scheme depends on fluorescence resonance energy transfer (FRET) to report the recognition of target analytes on glass bead supported lipid bilayers. Fluorescein and Texas red conjugated streptavidins assembled on lipid bilayers act as universal bearers of target biotinylated ligands and receptors. The diffusive encounter between ligand- and receptor-bearing streptavidins leading to recognition and attachment of the ligand/receptor pair, puts the FRET D/A pair in close proximity which triggers the FRET signal. Subsequently, a target analyte of higher affinity than the tethered pair is expected to dissociate the species thereby enabling the diffusive separation of the FRET donor acceptor pair and FRET is diminished. We have used this approach to display a series of ligands and receptors thus demonstrating the functionality of the *modular* components of the FRET scheme. We are currently attempting to resolve the remaining issues of optimization and geometrical constraints (lengths of tether of ligand receptor moieties with respect to R_0) of the ligand receptor pair. We have also used streptavidin coated polystyrene beads as platforms for the detection of FLAG epitope (DYKDDDDK) tagged proteins using a combination of biotinylated and fluorescently tagged peptides and antibodies. In this scheme, we have used flow cytometric methods to detect and quantify the binding of model FLAG epitope tagged proteins on beads with kinetic resolution. This detection scheme has the advantages of sensitivity, kinetic resolution, etc. over existing methods such as ELISA. In addition, the element of regeneration has been incorporated into the scheme when used with Ca^{2+} sensitive ligand receptor pairs (e.g. the FLAG peptide/M1 antibody pair): Where the addition and removal (with EDTA) of the cation can be used to regenerate the sensor. The results provide a general framework for understanding the factors which influence the practical needs of light-based sensors.

Key Words:
Fluorescence, Energy transfer, lipid bilayers, epitope tags.

Exhibit 5

Enclosure (3)

ANNUAL PROGRESS REPORT

01 June 1999 – 31 May 2000

GRANT NUMBER: N00014-95-1315

GRANT TITLE: Integrated Multi-Analyte Chemical Microsensors

PRINCIPAL INVESTIGATOR: Prof. G.P. Lopez (e-mail: gplopez@unm.edu)

PI INSTITUTION: University of New Mexico

AWARD PERIOD: 15 September 1995 to 14 September, 2000

REPORTING PERIOD: 01 June 1999 – 31 May 2000

OBJECTIVE: The broad objective of this work is to develop technologies that will enable the development of receptor-array based chemical sensors for the simultaneous monitoring of many chemical and biological analytes in aqueous environments. Specific aims toward this end include the development of: (1) model ligand-receptor systems; (2) reagentless molecular transduction systems based on fluorescence and electrochemical phenomena; (3) modular molecular assemblies that display the biomolecular systems described in 1 and 2 in a background of highly hidrated oligomers and polymers that inhibit nonspecific adsorpton and cellular attachment and thus allow sensing in complex aqueous environments; (4) methods for forming arrays of these modular molecular assemblies that allow the creation of multi-analyte sensor arrays; (5) optical instrumentation for highly sensitive, reliable, real-time transduction of these sensor arrays, including those based on established (i.e., SPR based) and new (i.e., fluorimetry- and electrochemistry-based) techniques; and (6) the application of data processing techniques for maximizing the information content and reliability of sensor output.

APPROACH: Our approach relies on a synergistic multi-disciplinary research team comprising researchers from the broad range of scientific fields necessary to tackle the important problem of reliable real-time detection and monitoring of multiple chemical and biological analytes in natural aqueous environments. These scientific disciplines include biochemistry, bioengineering, synthetic organic, analytical, physical and materials chemistry, chemical engineering, mechanical engineering, physics and optical engineering. Scientists and engineers from these disciplines have teamed into subgroups to address each of the specific objectives detailed above. Each of these subgroups include researchers from a wide range of levels of experience, including principal investigators, postdoctoral personnel, graduate students and undergraduate students. This teaming process has maximized the educational benefits of this research endeavor.

ACCOMPLISHMENTS (last 12 months):

- Development of a method for facile layer-by-layer synthesis of functionalizable, non-fouling assemblies on oxide and gold surfaces for biospecific interaction.
- Established protocols for incorporating Fab and $(Fab)_2$ antibody fragments into molecular assemblies, fluorescent tagging of proteins, and site specific biotinylation of whole antibodies (e.g. Fc portion labeled with biotinyl azide; attaching biotin to the SH hinge group on cleaved Fab fragments).
- Development of a reagentless method for detecting protein binding to surfaces based on incorporation of protein sensitive fluorophores into biospecific surface molecular assemblies.
- Fabrication of microchannels in elastomeric materials using photolithography and thick photoresists. A rapid prototyping protocol has been used to create microfluidic systems to pattern receptor proteins for SPR and fluorescence based array biosensors. These systems have also been used in the creation of microaffinity columns that can be monitored by a multichannel steady state/time domain detection system.

- Demonstration of a multichannel spectroscopic SPR imaging system for multi-analyte biosensing. Binding patterns of receptor arrays fabricated using microfluidic systems were studied.
- Study of diffusive mixing in microchannels of different geometries with the aim of developing methods for rapid mixing and reaction in low Reynolds number flows.
- Development of rapidly prototypable active microfluidic systems based on magnetically modified elastomeric materials and magnetic actuation circuits.
- Examination of geometrical constraints in development of molecular assemblies based on FRET and development of methods to resolve limitations of donor-acceptor proximity through overlabeling of receptors and optimization of tether lengths.
- Development of methods for displaying mobile receptors on lipid bilayer coated microspheres and demonstration of their activity. Receptor proteins include those used in construction of biomolecular assembies (e.g. avidin), antibodies, and cell surface receptors (e.g., ICAM-1).
- Development of a FRET / bead based assay to detect FLAG tagged proteins.
- Development of FRET based assays in microfluidic channels using flow cytometry calibrated beads as well defined platforms for molecular assembly and high surface area detection. Demonstrations included analysis of FLAG peptides in serum.
- Development of a robust and efficient synthetic methods for covalently linking DNA to glass and gold surfaces. This method provides enhanced reproducibility, uniformity and stability of immobilized DNA when compared to other approaches, especially those used to form DNA microarrays.
- Development of versatile microarraying instrumentation for the creation of two dimensional arrays of protein, DNA, and porous materials for use in multi-analyte sensing.
- Development of methods for rapid fabrication of patterned functional nanostructures via direct writing and printing.
- A new, viologen-containing, carboxylic acid-terminated thiol has been synthesized [$HSCH_2(CH_2)_9CH_2$-$\mathbf{V}^{2+}$-CH_2CO_2H, where **V** = 4,4'-bipyridyl, abbreviated to **VT**] in four steps and has been characterized by ^1H and ^{13}C NMR, and LR-MS. Monolayers of the **VT** on bulk gold electrodes showed poorly defined electrochemical response, which is probably due to disorder and poor packing of the **VT** in the monolayer surface.
- Electrochemical studies have been performed on the **VT** molecule in mixed SAMs with 11-hydroxyundecanethiol (**HUT**), diethylene-glycol-terminated undecanethiol (**EG-2**), triethylene-glycol-terminated undecanethiol (**EG-3**), and hexaethylene glycol-terminated undecanethiol (**EG-6**) on a bulk gold electrode; these studies show greatly improved electrochemical response compared to the pure **VT** monolayer. Non-specific adsorption of proteins to mixed monolayers of **VT** and **HUT** exhibit a significant (100 mV) shift in the redox potential for the viologen moiety; mixed monolayers composed of **VT** and **EG-3** did not exhibit non-specific adsorption of protein based on electrochemical data.
- Ternary SAMs composed of **VT** and a biotin-terminated thiol diluted with **EG-2**, **EG-3**, or **EG-6** have been prepared on a bulk gold electrode and characterized by electrochemistry, ellipsometry and XPS.
- Binding of antibiotin to these ternary SAMs results in a significant potential shift (>50mV) and a dramatic decrease in the current, but the binding of antibiotin is only partially reversible in the presence of free biotin. No measurable shift in the redox potential was observed when the ternary monolayers were exposed to biotin-blocked antibiotin. Parallel experiments have been performed utilizing SPR transduction which confirm the binding of antibiotin to the ternary monolayers and the incomplete dissociation of antibiotin in the presence of free biotin.These experiments demonstrate that it is possible to use voltammetric techniques to transduce the biospecific recognition of antibiotin with surface-bound biotin in a viologen-containing monolayer surface.
- Developed and implemented a phasefluorimetry-based multichannel detection system (sixteen channels, extensible to 63 channels) for fluorescence lifetime-based chemical/biological sensor arrays. The multichannel detection system has been tested with different types of chemical/biological sensor arrays (including chip-scaled arrays) and has been shown to be suitable for low resolution lifetime imaging.
- The multichannel detection system has been developed to monitor fluorescence lifetime changes in microfluidic channel-based chemical and biosensors and has been used to investigate laminar-flow-based diffusion mixing in microfluidic channels.

- Established the basis of a new lifetime-based flourescence detection technique which we call frequency fluorimetry. Designed, implemented, and tested an optoelectronic prototype for this detection technique for proof-of-concept purposes. The design is scalable for monitoring sensor arrays.
- Performed experiments to test new data processing techniques to increase the signal to noise ratio of the imaging-based Surface Plasmon Resonance array sensor platform.
- A fluorescence lifetime/intensity based scanning microscope has been designed and developed to image 2-D biosensor arrays. The unit has a spatial resolution down to diffraction limit and sensitivity down to single molecule levels. The field of view can be varied from few microns to several millimeters depending on the application. Image acquisition time per pixel can be as short as 100 micro seconds. Monolayer sensor arrays based on Alexa fluorophore labeled proteins could detect the presence of a test analyte, as seen from the lifetime images acquired by the unit
- The relaxation dynamics of surface plasmons in gold were studied in order to improve the understanding of surface plasmon interaction with the local environment for sensor applications. Surface plasmon resonance was used as a sensitive technique to detect pump induced changes in the dielectric constant of the order of 10^{-4}. The use of surface plasmon resonance allows discrimination between changes in the real and imaginary part of the dielectric constant and therefore allows to monitor changes in the imaginary part directly. The thermalization of the excited electron gas has been observed directly. The electron-phonon relaxation has been monitored.

SIGNIFICANCE: This work will enable (1) field-deployable instrumentation for the simultaneous detection and monitoring of multibple aqueous analytes in real time; and (2) the development of new analytical methods for study of biomolecular and cellular interactions with synthetic surfaces.

WORK PLAN (next 3 months): In the final few months of this research program, we will complete the demonstrations and testing of the sensors systems described above to fulfill the objectives outline in the original and renewal proposals.

PUBLICATIONS, ABSTRACTS, TECHNICAL REPORTS, PATENTS, AND AWARDS (last 12 months):

Publications

"Rapid Prototyping of Active Microfluidic Components Based on Magnetically Modified Elastomeric Materials," Jackson, W.C.; Tran, H.D.; O'Brien, M.J.; and López, G.P. *J. Vac. Sci. Tech. B* (accepted pending revision).

"The Use of Self-Assembled Monolayers (SAMs) of Different Wettability to Study Surface Selection and Primary Adhesion Processes of Zoospores of the Green Alga *Enteromorpha*," Callow, M.E.; Callow, J.A.; Ista L.K., Coleman. S.E.; Nolasco. A.C.; and López, G.P. *Applied and Environmental Microbiology* (in press).

"A Generalizable Fluorescence Biosensing Strategy Based on Energy Transfer Between a Metal Surface and Fluorescently-Labled Receptors," Perez-Luna, V.H; Yang, S.; Rabinovich, E.; Hampton, P.D.; López, G.P. *Anal. Chem.* (submitted).

"A Surface Plasmon Resonance Array Biosensor Based on Spectroscopic Imaging,", O'Brien, M.J.; Pérez-Luna, V.H.; Brueck, S.R.J.; López, G.P.; *Biosensors and Bioelectronics* (submitted).

"Rapid Prototyping of Patterned Functional Nanostructures," Fan, H.; Lu, Y.; Stump, A.; Reed, S.T.; Baer, T., Schunk, R.; Prez-Luna, V.; López, G.P.; Brinker, C.J. *Nature,* **2000**, *405*, 56-60.

"Phase-sensitive Multichannel Detection System for Chemical and Biosensor Arrays and Fluorescence Lifetime-based Imaging," Rabinovich, E., O'Brien, M.J., Brueck, S.R.J., and López, G.P. *Rev. Sci. Instr.,* **2000**, *71,* 522-529.

"Molecular Recognition Between Genetically-Engineered Streptavidin and Surface Bound Biotin," Pérez-Luna, V.H.; O'Brien, M.J.; Opperman, K.A.; Hampton, P.D.; López, G.P. *J. Am. Chem. Soc.,* **1999**, *121,* 6469-6478.

"Multifunctional Monolayer Assemblies for Reversible Direct Fluorescence Transduction of Protein-Ligand Interactions at Surfaces," Sekar, M.M.A.; Hampton, P.D.; López, G.P. *J. Am. Chem. Soc.*, **1999**, *121,* 5135-5141.

"Peptides, Antibodies, and FRET on Beads in Flow Cytometry: A Model System Using Fluorescienated and Biotinylated β-Endorphin," Buranda, T.; López, G.P.; Keij, J.; Harris, R.; Sklar, L.A. *Cytometry*, **1999,** *37,* 21-31.

"Surface-Grafted, Environmentally Sensitive Polymers for Biofilm Release," Ista, L.K.; Perez-Luna, V.H.; López, G.P. *Appl. and Environ. Microbio.*, **1999**, *65*, 1603-1609.

"Ligand-Receptor Dynamics at Streptavidin Coated Particle Surfaces: A Flow Cytrometric and Spectrofluorimetric Study," Buranda, T.; Jones, G.; Nolan, J.P.; Keij, J.; López, G.P.; Sklar, L.A. *J. Phys. Chem.*, **1999**, *103*, 3399-3410.

"Enhancing IR Detection Limits for Trace Polar Organics in Aqueous Solutions With Surface-Modified Sol-Gel-Coated ATR Sensors, Han, L;. Niemczyk, T.M.; Halland, D. M; López, G.P. *Appl. Spectroscopy*, **1999,** *53*, 381-389.

"Surface Plasmon Resonance Measurement of Binding and Dissociation of Wild-Type and Mutant Streptavidin on Mixed Biotin-Containing Alkylthiolate Monolayers," Jung, L.S.; Nelson, K.E.; Campbell, C.T.; Stayton, P.S.; Yee, S.S.; Perez-Luna, V.H.; López, G.P. *Sens. and Actuat. B*, **1999**, *54*, 137-144.

"SPR Biosensors: Simultaneously Removing Thermal and Bulk Compositional Effects," O'Brien, M.J.; Brueck, S.R.J.; Perez-Luna, V.H.; Tender, L.M.; López, G.P. *Biosens. & Bioelec.*, **1999**, *14*, 145-154 .

"Two-Dimensional Patterning of Proteins," Yang, S.; Perez-Luna, V.H.; López, G.P. In *Protein Architecture: Interfacing Molecular Assemblies and Immobilization Biotechnology,* Marcel Dekker, 1999.

"Nanotechnology," Vaidya, R.; López, G.P.; López, J.A. In *Kirk and Othmer's Encyclopedia of Chemical Technology*, John Wiley and Sons (in press).

"Nanotechnology," Vaidya, R.; López, G.P.; López, J.A. In: *The Concise Encyclopedia of Chemical Technology, 4th Ed.,* pp. 1341-1343. John Wiley and Sons, 1999 (this chapter is a condensed version of the one listed above).

"Patterned Functional Arrays by Selective De-Wetting," Fan, H.; Doshi, D., Lu, Y., López, G.P., and Brinker, C.J. In *Mat. Res. Soc. Symp. Proc.*, Vol. 628, **2000**, CC6.33.1-CC6.33.6.

"Surfactant Templated Mesoporous Hybrid Thin Films," Fan, H.; Lu, Y.; Assink, R.A.; López, G.P., and Brinker, C.J. In *Mat. Res. Soc. Symp. Proc.*, Vol. 628, **2000**, CC6.41.1-CC6.41.6.

"Compact Phase-Sensitive Multichannel Detection System with Array Measurements of Biosensor Chips," Rabinovich, E.M.; O'Brien, M.J.; Brueck, S.R.J.; Yang, S; Pérez-Luna, V.H.; López, G.P. In *Progress in Biomedical Optics,* Proc. of SPIE, **2000**, *1*, 181-185.

"Hierarchically Structured Functional Porous and Composite Mesostructures Produced by Evaporation-Driven Self-Assembly," Fan, H.; Reed, S.T.; Baer, T.; Schunk, R.; Lopez, G.P.; Brinker, C.J. *Micro. Meso. Mater.*, in press.

"Sheath Fluid Control to Permit Stable Flow in Rapid Mix Flow Cytometry," Seemer, L.C.; Kuckuck, F.; Sklar, L.A. *Cytometry,* **1999**, *35,* 75-79.

Abstracts

"Reagents, Molecular Assemblies, and Instrumentation for Multi-Analyte Biosensor Arrays," López, G.P. Invited seminar presented at The University of Washington, Department of Bioengineering, Seattle, Washington, June 2, 1999.

"Studies on Biofilm Resistance, Formation and Release on Model Solid Surfaces," López, G.P. Presentation at the 1999 ONR Biofouling and Hull Coatings Review, Arlington, Virginia, August 2, 1999.

"Molecular Thin Film Assemblies for Reversible Fluorescence-Based Biosensors," López, G.P. Presentation at the American Institute of Chemical Engineers 1999 Annual Meeting, Dallas, Texas, November 5, 1999.

"Strategies and Instrumentation for Optics-Based Biosensor Arrays," López, G.P. Invited Presentation at the Defense Advanced Research Projects Agency Workshop on Biophotonics, Arlington, Virginia, March 14, 2000.

"Streptavidin in Molecular Assemblies for Optics-Based Biosensors: Implications and Applications," Lopez, G.P., Buranda, T. Sklar L.A. Invited Presentation at the 1st International Conference on (Strept)Avidin-Biotin Technologies, Banff, Alberta CA, June 18-21, 2000.

"Rapid Fabrication of Patterned Functional Nanostructures via Direct Writing and Printing," Fan, H.; Lu, Y.; Reed, S.T.; Baer, T.; Schunk, R.; Lopez, G.P.; Brinker, C.J. Presented at the Annual Meeting of the Materials Research society, April 24-28, 2000, San Francisco.

"Organic-Inorganic Multifunctional Mesostructured Silica Films," Fan, H.; Lu, Y.; Reed, S.T.; Baer, T.; Schunk, R.; Lopez, G.P.; Brinker, C.J. Presented at the Annual Meeting of the Materials Research society, April 24-28, 2000, San Francisco.

"Rapid Fabrication of Patterned Functional Arrays," Fan, H.; Lu, Y.; Lopez, G.P.; Brinker, C.J. Presented at the Annual Meeting of the Materials Research society, April 24-28, 2000, San Francisco.

"Rapid Prototyping of Patterned Functional Arrays," Fan, H.; Lu, Y.; Lopez, G.P.; Brinker, C.J. Presented at the Annual Meeting of the American Chemical Society, August 20-24, 2000, Washington D.C.

"Molecularly Imprinted Sol Gel Films for Analyte-Selective IR Waveguide Sensors," Haibach, F.G.; Niemczyk, T.M. Presented at the Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy 50th Annual meeting, New Orleans, LA; March 2000

"Compact Phase-Sensitive Multichannel Detection System with Array Measurements of Biosensor Chip," Rabinovich, E.M.; O'Brien, M.J.; Brueck, S.R.J.; Yang, S.; Perez-Luna, V.H; Lopez, G.P., Presented at BiOS 2000, January 22-28, 2000, San Jose, CA.

"A Phase-Fluorimetry-Based Multichannel Detection System for Chemical and Biological Sensor Arrays," Rabinovich, E.M.; O'Brien, M.J.; Brueck, S.R.J.; Yang, S.; Perez-Luna, V.H; Lopez, G.P., Presented at "Optoelectronics, Photonics, and Imaging," April 10-11, 2000, Albuquerque, NM.

"Biosensors based on laser scanning microscope," Nampoothiri, A.V.V; Ukhanov, A.; Rudolph, W. Presented at 6th Annual Symposium of the New Mexico chapter of the American Vacuum Society, Albuquerque, USA, May 22-26, 2000.

"Femtosecond Surface Plasmon Dynamics in Metal Films for Sensor Applications", Stock, R.; Nampoothiri, A.V.V.; Lopez, G.P.; Rudolph, W. SPIE conference, April 10-11, 2000, Albuquerque, pp 38-39

"Investigation of Electron Thermalization using Surface Plasmon Resonance as Optical Probe", Stock, R.; Nampoothiri, A.V.V.; Lopez, G.P.; Rudolph, W. Lasers 2000 proceedings (accepted for presentation).

"Multianalyte Sensors on Streptavidin-Bearing Beads" Buranda, T.; Lopez, G.; Sklar, L. Presented at the First International Conference on (Strept)avidin-Biotin Technologies, Banff Centre, Alberta, Canada June 18-21, 2000.

"Reagentless and Regenerable Sensors on Beads" Buranda, T.;Robledo, T.; Lopez, G.; Sklar, L. Presented at the Sixth World Congress on Biosensors, San Diego, May 24-26, 2000.

"A Surface Plasmon Resonance Imaging System for Multianalyte Biosensing Applications", Perez-Luna, V.H.; O'Brien, M.J.; Lopez, G.P. Presented at the Annual Meeting of the American Institute of Chemical Engineers, Oct. 31 – Nov. 5, 1999, Dallas, TX.

"Rapid Prototyping of Microfluidic Components," Jackson, W.; Tran, H.D.; Lopez, G.P. Presented at the Annual Meeting of the American Vacuum Society-New Mexico Chapter, May 22-26, 2000, Albuquerque, NM.

Patents and Patent Disclosures

"Materials and Methods for Rapid Prototyping of Active Microfluidic Systems," Tran, H.D.; López, G.P.; Jackson, W.C. Patent application submitted.

"Robust and Efficient Synthetic Method for Covalently Linking DNA to Oxide and Metal Surfaces", Wu Y.; Dolan, P.; Nelson, M.A., Lopez, G.P., Patent disclosure.

"Rapid Prototyping of Patterned Functional Nanostructures," Fan, H.; Lu, Y.; Perez-Luna, V.; López, G.P.; Brinker, C.J. Patent application submitted.

"Simple and Inexpensive Optoelectronic Auto-Oscillatory Circuit for Fluorescence Lifetime-Based Chemicl Biological Sensor and Sensors Array Monitoring." O'Brien, M.J. Rabinovich, E.M; Brueck, S.R.J.; Svimonishvili, T.; Lopez, G.P. Patent disclosure.

Exhibit 6

Office Use Only:	
Docket Number: MC-193	Prior or Pending Publication: yes
Completed Disclosure Date: 2/7/01	Non-governmental Sponsored Research: no

Govt Sponsored yes

CONFIDENTIAL

UNIVERSITY OF NEW MEXICO INVENTION DISCLOSURE FORM

NOTE: ALL SECTIONS MUST BE COMPLETED BEFORE PROCESSING CAN BEGIN. Inventor(s) must also execute the attached assignment. For assistance with or questions regarding Section V, or in an emergency due to an imminent publication or presentation, contact Science & Technology Corp. @ UNM (STC) at 272-7900; for other questions or assistance contact the Patent Administration Office (PAO).

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First Name

The Lead Inventor (who may or may not be the principal inventor) is responsible for:
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(2) providing requested documents to PAO, STC, and the patent attorney; and
(3) notifying STC and the patent attorney of changes in invention status.

I understand and agree to comply with the responsibilities set forth above as Lead Inventor for this invention.

Tione Buranda 2/9/01
Signature Date

Invention Title: *Porous affinity sensor with fluorescence detection*

Initial disclosure submission date: 11/8/00 (MC193)

Rec'd STC 4/24/01

III. INVENTION DETAILS:

A. Date of conception of the invention: 6/1999_____
How was this documented (e.g. lab notebook, computer disk, photograph, chart)?
Original Pictures, of beads in microchannels, Manuscript

B. Please attach a **detailed** description of the invention on a separate page.

C. Has the invention been reduced to practice (e.g. experiments, proof of principle, prototypes, animal studies, clinical trials)? If so, when and how:
Yes (see attached manuscript)

IV. PUBLICATIONS / DISCLOSURES

A. Inventors' publications on this invention as of the date of this disclosure*:
1. NA_____ Publication Date:_____
2._____ Publication Date:_____
3._____ Publication Date:_____
4._____ Publication Date:_____

B. Anticipated inventors' publications on this invention*:
1.Rapid and Quantitative Detection of Biomolecules on Calibrated Beads in Microfluidic Channels. Date Submitted _____ Expected Pub. Date:_____
2._____ Date Submitted _____ Expected Pub. Date:_____
3._____ Date Submitted _____ Expected Pub. Date:_____
4._____ Date Submitted _____ Expected Pub. Date:_____

*Please submit a copy of any publications to the Patent Administration Office with this form.

C. Please include the details (including date, subject matter, and meeting or audience) of any presentations or talks regarding the invention made to non-University personnel (attach a copy of the slides or other materials used, if any):

1. "Multianalyte Sensors on Streptavidin-Bearing Beads" Buranda, T.; Lopez, G.; Sklar, L. (Abstract attached) L. First International Conference on (Strept)avidin-Biotin Technologies, Banff Centre, Alberta, Canada June 18-21, 2000. (Abstract attached)

2. "Reagentless and Regenerable Sensors on Beads" Buranda, T.; Robledo, T.; Lopez, G.; Sklar, L. Sixth World Congress on Biosensors, San Diego, 24-26 May 2000.
3. Presentation by Lopez to ONR on Research progress 11/9/00 (slides given to STC for provisional file)

Exhibit 7

Rapid Quantification of Biomolecular Recognition on Calibrated Beads in Microfluidic Channels

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ABSTRACT

We have developed a new approach for the analysis of biomolecular recognition in microfluidic channels. The method is based on real-time detection of biomolecular binding to receptor-bearing microspheres comprising affinity microcolumns. Model assays demonstrated, include direct fluorescence methods of quantitatively detecting recognition of model analytes by protein receptors and ligands displayed in well-characterized affinity matrices. We establish a model system for detection of recognition between a monoclonal antibody and the FLAG™ epitope tag. The assay can potentially detect sub-femtomole quantities of antibody with high signal-to-noise ratio and a large dynamic range spanning nearly four orders of magnitude in analyte concentration. Kinetic and equilibrium constants for the reaction of this receptor-ligand pair are obtained through modeling of kinetic responses of the microcolumn and are consistent with those obtained by flow cytometry. Because of the correlation between kinetic and equilibrium data obtained for the microcolumns, quantitative analysis can be done in minutes, prior to the steady state endpoint of the recognition reaction. The approach has the potential to be generalized to a host of bioaffinity assay methods including analysis of protein complexes and molecular assembly and microsystem-based multi-analyte determinations.

Key Words: microfluidics, biosensors, proteomics, flow cytometry, beads, fluorescence, energy transfer

INTRODUCTION

The analysis of macromolecular interactions, assemblies, and their function is an essential component of biomedical research. This effort is driven by identification of diagnostic or therapeutic targets of disease, analysis of ligand binding and enzyme kinetics, DNA sequence detection or analysis,[1] and biosensing.[2-4] Important progress in the development of new technologies for biomolecular analysis has been in the area of microfluidic devices.[5] Microfluidic devices generally consume sub-microliter quantities of sample and are thus well suited for use when the required reagents are scarce or expensive. Because of their size, microfluidic devices operate in a regime where small Reynolds numbers[6] govern the delivery of fluid samples. Fast mixing of reagents is one of several issues that present a major challenge to the operation of microfluidic devices. Due to negligible inertial forces,[6] mixing of solutes in microchannels is as a rule driven by diffusion alone,[7-9] and is therefore slow and often ineffective even at micrometer scales. Other factors including fluid transport and quantitative analysis (chemical reaction, product separation and identification) of molecular interactions are poorly understood and must be optimized to fully realize the potential of these micro-devices.

A prevailing trend in the development of bioanalytical assays, including those incorporating combinatorial methods,[10] is the display of biochemical reagents on synthetic microbeads.[11, 12] Commercial sources (e.g. Spherotech, IL; Bangs, IN, Polysciences; PA) for bead-based display systems (e.g., covalent coupling, biotin-streptavidin, His-tag) are currently available. Concurrent with this development has been the advances in molecular approaches to incorporating fluorescent tags to proteins as well

as bead based surface chemistries and calibration approaches that allow the quantitative study of these molecular interactions by flow cytometry. [13, 14] For several years we have participated in this trend and thus have extensive experience in developing biosensing strategies that display functional, fluorescently labeled receptors and ligands on microbeads.[14-16] Through this endeavor, we have shown how molecular assemblies on beads can be analyzed in a quantitative fashion by flow cytometry.

We have used beads calibrated with flow cytometry,[14] as platforms in an affinity micro-column format for the quantitative detection of analytes in microfluidic channels (Figure 1). There are several advantages of this approach. Molecular assemblies for the assay are created outside the channel on beads and calibrated with flow cytometry. Uniform populations of beads may be insured through rapid cytometric sorting. Beads present a larger surface area for the display of receptors than flat surfaces. This is a clear improvement over those techniques that rely on the micro patterning of reactive molecules on flat surfaces.[17] Mixing of solutes in laminar flows occurs by diffusion. The typical diffusion coefficient for biomolecules is on the order of $\leq 10^{-7}cm^2s^{-1}$ thus mixing by diffusion is slow. Rapid mixing in the microcolumn is achieved because the distance that must be covered by diffusion is limited to the (small) interstitial space between the closely packed receptor-bearing beads. Analytes are captured in flow-through format thus each bead can act as a local concentrator of analytes.[18] Beads can be easily configured to detect multiple analytes in the restricted confines of a microchannel. Simultaneous detection of a diverse group of analytes can be achieved by packing, discrete segments of receptor bearing beads in a single affinity micro-column (work in progress).

This report describes the detection of analytes and the determination of kinetic and equilibrium constants of binding between biomolecules in microchannels. Samples of Texas Red labeled antiFLAG™ monoclonal antibodies (TR-M1 mAbs) were pumped through an affinity microcolumn with fluorescein-tagged FLAG peptides on beads with known site densities. The interaction between the TR-M1 mAbs and beads was monitored via fluorescence resonance energy transfer (FRET).[19] Monitoring the amount of ligand/receptor complex formed at a wide range of concentrations of TR-M1 mAbs gave access to the kinetic and equilibrium parameters of the antibody–peptide reaction. The data from affinity micro-columns were compared to data measured in a conventional flow cytometer assay.

Our ultimate goal is to develop generalized nanofluidic affinity matrices composed of flow cytometry certified microbeads bearing the biomolecular assemblies necessary for the precise, specific and sensitive detection of chemical and biological analytes. Because it is possible to study the dynamics of ligand-receptor interactions on the beads with flow cytometry, similar experiments in microchannels will enable researchers to delineate the limits of microfluidic perturbation.

RESULTS AND DISCUSSION

We have previously used a combination of flow cytometry and spectrofluorimetry to analyze macromolecular interactions on the surfaces of microspheres.[14] The work was motivated by the need to develop bead-based methodologies for high throughput proteomic[3] assays. Herein, surface calibrated beads are sequestered in microfluidic channels (Figure 1) and used as platforms for the dynamic and quantitative detection of

biomolecules at sub-microliter volumes. The microfluidic channels were made from an elastomeric polymer, poly(dimethylsiloxane) (PDMS), where convenient fabrication techniques allow for dimensions as small as 10 μm.[5] The prototype shown in Figure 1 is composed of a microfluidic channel, 3 cm long, with typical dimensions of 250μm by 50μm in breadth and depth, patterned into a PDMS elastomer adhered to a glass slide support. Within the microchannel, obstructive features 20μm apart were patterned as filters to hold 30μm beads. Beads were packed by injection of suspensions, starting with a foundation of 30μm borosilicate beads followed by the affinity micro-column layer of thirty thousand, 6.2μm streptavidin coated beads. The streptavidin coated beads bore biotinylated molecules of interest. A useful dimension of the active microcolumn is the volume of bead interstitial space (≈ 4.0nL for the 600μm long column) that serves as a reactor vessel with an intrinsically large surface area.

We have selected biotin (MW = 244.3 *g*/mole) and a monoclonal antibody (MW ≈ 150,000 g/mole) as prototypical analytes representative of small and large molecules respectively. The equilibrium and kinetics of binding and dissociation of these two systems have been characterized and are reasonably well understood.[20-23] This enables us to make comparisons of quantitative data collected with flow cytometry and micro-columns. The presence of the analytes is monitored in real time by fluorescence intensity increases for detection of biotin, and FRET for the detection of Texas Red labeled monoclonal antibody. A salient feature of this analysis is that the initial intensity of the beads corresponds to a known concentration of surface receptors. Thus, the subsequent changes from the initial intensity reading bear definite and known relationships to the amount of captured analytes, without contribution from unbound species.

Detection of Native Biotin Via Fluorescence Unquenching of Fluorescein Biotin. It has been previously shown that under certain circumstances, binding of fluorescent ligands to streptavidin is characterized by the quenching of fluorescence of bound relative to free ligands.[18] Typically, this type of quenching ("ostrich quenching") occurs when the fluorophore (e.g., fluorescein) moiety of a biotinylated ligand associates with the receptor pocket adjacent to the biotin-moiety bearing site (Figure 2). Ostrich quenching is dependent on the length and stereochemistry of the ligand. We have previously shown this interaction for fluorescein biotin to be very weak ($K_d \approx 0.1$),[18] and readily obstructed by native biotin.[14, 24, 25] The binding of fluorescein biotin to excess soluble streptavidin results in >90% quenching of the fluorescence. Addition of native biotin recovers the original intensity under diffusion-limited kinetics. The extent of quenching and recovery on beads depends on site occupancy of the fluorescein biotin. The data in Figure 2B shows a fivefold increase in intensity of fluorescein biotin-bearing beads resulting from the elution of a 2μL aliquot of 3 mM native biotin. The five-fold increase in intensity was consistent with a result from a flow cytometry measurement (data not shown). The data shows good signal to noise ratio (n_1). The magnitude of n_2 relative to n_1 is likely due to the disruption of the packing of beads upon initial contact with the plug of sample. It is likely that such disruption can be minimized through optimization of bead packing and sample injection procedures.

The transport-limited kinetics and high affinity ($k_{off} \approx 10^{-6} s^{-1}$) of the binding of biotin/streptavidin[20] allows us characterize the fluid flow properties inside the channel. The volume of the reactor vessel is comprised of interstitial space between the receptor bearing beads. The average time for a molecule to diffuse across a distance d is $t = d^2/2D$

where D is the diffusion coefficient of the molecule. In the column, d is small. The time lapse for diffusive contact between the biotin and receptor surface is correspondingly small. Because of the fact that the biotin is in large excess of the streptavidin receptors, the leading edge of the fluid passes through the column with negligible depletion of biotin. A direct correlation can therefore be made between the time-resolved increase in intensity and the velocity of the fluid. Based on the data in Figure 2B, the flow rate through the column (≈ 4.0 nL interstitial volume) is on the order of 1.6 nLs^{-1}. Because the biotin experiment is essentially irreversible and simple it serves as a useful calibration standard of the affinity micro-column, and facilitates the analysis of the more complex antibody binding data below.

Detection of Anti-FLAG Monoclonal Antibodies Via FRET in Microchannels. Epitope tagging is a widely practiced technique used to purify and study structure and functional properties of proteins.[23, 26-28] One of the most common epitope tags is the FLAG™ system that relies on the FLAG™ octapeptide (DYKDDDDK) fusion tag that has readily available monoclonal antibodies. We have chosen this system as a model for development of generalizable assays for proteins with known epitopes. We have synthesized biotinylated and fluorescein tagged FLAG™ peptides, attached them to streptavidin-coated beads (≈ $1x10^6$ peptides/bead) and used FRET to analyze their interaction with Texas Red labeled anti-FLAG (TR-M1) monoclonal antibodies (mAbs) by flow cytometry.[23] Results from that study are compared to the analytical data collected in the affinity micro-columns described here. Several concentrations of TR-M1 mAbs were analyzed with affinity micro-columns. The results are shown in Figure 3. The passage of TR-M1 mAbs through the affinity micro-column of FLAG™ peptide-bearing

beads was monitored by the (FRET) quenching of the fluorescein tagged FLAG™ peptide fluorescence. The theory and practice of FRET are well understood and documented.[19, 29, 30] The level of FRET efficiency depends on the average donor acceptor distance. In order to optimize the incidence of FRET resulting from peptide/antibody binding, antibodies were fluorescently labeled with high levels of the Texas Red averaging ≈ 6.0 tags/antibody. [29]

In Figure 3A, various concentrations of TR-M1 were eluted through the column at the rate of 1.6nLs^{-1}. Data were normalized to the initial intensity of the beads before passage through the column of a 2μL aliquot of TR-M1 mAbs. The data in Figure 3A were fit to a kinetic model shown in Equation 1, expressed in terms of bimolecular interactions and diffusion-limited conditions. [31]

$$\frac{d\Gamma_{AB}}{dt} = k_f C_s \Gamma_A - k_b \Gamma_{AB} = \frac{D}{\delta}(C^* - C_s) \qquad (1)$$

C^* and C_s represent the concentrations of antibody in the bulk and at the liquid-solid interface respectively; Γ_{AB} is the surface concentration of FLAG™ peptides bound to antibodies; Γ_A is the surface concentration of unbound peptides; and k_f and k_b are the forward and reverse kinetic rate constants. D is the diffusion coefficient of the antibody and δ is the thickness of the steady-state diffusion-convection boundary layer established by fluid transport in which we assume a linear gradient in concentrations (between C^* and C_s). The parameter D/δ represents the effects of diffusive transport of analytes to the surface receptors. The integral form of this equation is:

$$\lambda\theta - \left(1 + \frac{\lambda}{1+\kappa}\right)\ln\left(1 - \frac{1+\kappa}{\kappa}\theta\right) = \frac{1+\kappa}{\kappa}\lambda\tau \qquad (2)$$

In this equation $\theta = \Gamma_{AB}/\Gamma^s$, where Γ^s ($=\Gamma_{AB}+\Gamma_A$) represents the total surface concentration of FLAG™ peptides. The equation is composed of dimensionless parameters: the binding equilibrium constant, $\kappa = \frac{k_f}{k_b} C^b$, the diffusion dependant rate of adsorption, $\lambda = \frac{k_f \delta \Gamma^s}{D}$, dimensionless time normalized to diffusion time, $\tau = \frac{t}{t_d}$, and the time that characterizes the diffusion process, $t_d = \frac{\delta \Gamma^s}{D C^b}$. Least squares error minimization[33] between this equation and experimental data was performed with a Nelder-Mead Simplex algorithm. [33] For a 6.2 μm diameter bead with 10^6 receptors per bead, $\Gamma^s = 1.38 \times 10^{-10}$ mol/dm^2. It is important to note that disperaion of the sample plug and depletion (especially with dilute samples) of TR-M1 mAbs occurs which results in a gradient of Γ_{AB}. Because the detection of the binding process is intergrated over the whole column a simplifying assumption that negates gradients is made. The fits to the experimental data yield the following parameter values: $K_d = 13.3 \pm 2.0$ nM, $k_f = (9.0 \pm 6.0) \times 10^6$ M s^{-1}, $k_b = (1.2 \pm 0.8) \times 10^{-3}$ s^{-1}, $D/\delta = (1.0 \pm 0.9) \times 10^{-9}$ dm s^{-1} where the errors are the standard deviations for the constants determined from the fittings of each experimental run. The kinetic data are on the same order of magnitude with data reported in the literature on similar antibody-antigen interactions.[32] Figure 3B shows the analysis of data collected after the elution of 2μL plugs of TR-M1 was complete. The equilibrium dissociation constant (K_d) from this analysis is ≈10.0 nM, which is in close agreement to the K_d derived from the kinetic model, $K_d = k_b/k_f$=13.3 nM. The conservation of microscopic reversibility, as shown by this close correlation of steady state and kinetic

data indicates that it is possible to make fast determinations of soluble analytes based on the dynamic response of the affinity micro-column.

In order to corroborate the analysis of the affinity micro-column data, we turn to flow cytometry. Figure 4 is a sigmoidal dose response curve of flow cytometry data[22] using FLAG™ peptide-bearing beads and TR-M1 mAbs taken from the same stock as the reagents used herein. The flow cytometer readings were taken after 30 minute incubations of beads with TR-M1 mAbs. In comparison, the affinity micro-column data were read after the elution of the 2μL volume of mAbs (≈ 21 min). The flow cytometer intensity readings derive from the mean channel fluorescence[14, 23] of bead-borne peptide fluorescence after binding of TR-M1 mAbs. The data are normalized to the intensity before FRET quenching which results from antibody binding. The dissociation constants determined from flow cytometry (4.0 nM), and the affinity micro-columns (10.0 nM) are in good agreement. Although the differences in the dissociation constants are within experimental error, it is possible that the K_d derived from the affinity micro-column data is larger because a portion of interstitial bead space was not accessible to the flowing TR-M1 mAbs, thus leaving a small percentage of the peptide unexposed to mAbs.

Prospects for Analytic Methods. In this work we have shown that flow cytometry characterized beads in microfluidic channels are viable platforms for dynamic quantitative analysis. There are several important features of this assay worthy of emphasis: *sensitivity, response, dynamic range and economy.* In the current configuration, it is possible to detect femto-mole range of protein (0.48nM – 4.8 nM curves in Figure 3). The high signal to noise ratio of these assays is due to the fact that the analytes are dark (biotin) or do not contribute any background (TR-M1) to the change

in the fluorescence of the fluorescein tag. The assay has a wide dynamic range spanning nearly four orders of magnitude of analyte concentration. The good correlation between kinetic and equilibrium data enables one to determine concentrations of analytes from dynamic response; thus assays can be carried out in a few minutes, supplanting the need for time consuming steady state endpoint assays. In preliminary experiments, we have determined that the detection of TR-M1 and a FRET blocking non-fluorescent peptide can be achieved in a controlled manner in an analyte fluid comprised of blood serum and buffer. This indicates that this assay format is amenable to clinical applications.

Multianalyte Detection. We are currently developing a multi-analyte model system comprised of discrete segments of beads that bear distinct receptors for the simultaneous detection of diverse analytes (Figure 5). Since these assays consume very small sample volumes, multiple tests can be run, therefore saving on expensive reagents.

EXPERIMENTAL PROTOCOL

Materials. 6.2 μm diameter streptavidin-coated polystyrene beads (Spherotech Inc., Libertyville, IL) were obtained as 0.5% (w/v) suspensions according to the manufacturer's data sheet. Hemocytometer analysis of the beads revealed the concentration of the particles to be in the range of $4\text{-}5\times10^7$ beads/ml depending on the lot. Biotin, fluorescein biotin (5-((N-(5-(N-(6-(biotinoyl)amino)hexanoyl)amino)pentyl)thioureidyl)-fluorescein) and (6-((6-((biotinyl)amino)hexanoyl)amino)hexanoic acid, succinimidyl ester (biotin-XX, SE) were purchased from Molecular Probes (Eugene, OR) and used without further purification. Anti-FLAG antibody was purchased from Sigma Chemical (St. Louis, MO). Biotinylated

and fluoresceinated FLAG peptides (biotinyl-ε-amino caproyl-KK(fluorescein)KDDDDKYD-NH_2 and biotinyl-ε-amino caproyl-KKDDDDKYD-NH_2) and K(fluorescein)KDDDDKYD-NH_2) were synthesized at the University of New Mexico Protein Chemistry Laboratory.

Determination of Affinities of Fluorescent Ligands Binding to Beads via Fluorescence and FRET Analyses. Experimental details of these analyses have been described elsewhere,[14, 24] however, brief descriptions are provided for clarity. Centrifugation assays were used to determine the binding capacity of biotinylated fluorescent ligands, to beads where the amount of bound ligand was determined from the fluorescence intensity of the residual supernatants using an SLM-Aminco 8000 spectrofluorimeter (SLM Instruments, Rochester, NY). The resuspended beads were analyzed by flow cytometry using a Becton-Dickinson FACScan flow cytometer (Sunnyvale, CA). The average fluorescence on a single bead is converted to the number of fluorophores per bead on the basis of flow cytometric calibration beads.[14, 24] Binding affinity of biotinylated ligands for their receptor antibodies was determined in solution or on beads using FRET. [22]

Preparation of Microchannels. The microfluidic channels were made from an elastomeric polymer, PDMS, following published methods[5] employing standard photolithographic techniques.

Packing of Microchannels with Beads. In a typical experiment, a 30μL aliquot suspension of 30 μm beads (≈ 10^6 beads/mL) was injected into the channel (Figure 1) with a Hamilton syringe. With the filter acting as a barrier to bead flow-through, the supernatant was gently removed from the channel with the aid of a peristaltic pump. This

was followed by a 30 μL volume of fluorescent ligand-bearing polystyrene beads (≈ 1.0 x 10^6 beads/mL or ≈ 30,000 beads in the micro-channel) in Tris buffer (pH = 7.5) containing 0.1% BSA. The column was allowed to settle during the peristaltic pump-assisted elution of several microliters of buffer. The elution of buffer through the column also allows for the coating of the PDMS micro-channel with BSA, thus minimizing the potential for non-specific adsorption of protein and peptides to the walls of the microchannels in subsequent assays. Once packed, the column was ready for use. In this work we used streptavidin-coated beads, which bore known quantities of either fluorescein biotin or fluorescent FLAG peptide. Their respective 'analytes' comprising native biotin or Texas red tagged anti FLAG antibodies were injected into the channel in 2μL aliquots. The time resolved interaction between the analyte solutions and beads was monitored on a Model Fluorolog-3 SPEX fluorimeter (Instruments S.A.; Edison NJ) using 488 nm laser excitation focused onto the 0.6mm long portion of the column comprised of the fluorophore-bearing beads.

Fluorescein Biotin Column. Streptavidin coated beads bearing ≈ 1 x 10^6 fluorophores/bead were packed into the micro-channel *(vide supra)*. The analyte fluid (3.0mM biotin in 2μL) was added to the column and monitored as increasing emission intensity of the beads as the fluid flowed through the column.

Fluorescent FLAG Peptide Bearing Beads. Several affinity micro-columns were prepared using ≈ 1.0 x 10^6 peptides/bead.[22] 2μL plugs of Texas Red labeled M1 anti-FLAG monoclonal antibodies (TR-M1) were eluted at varying concentrations in different affinity micro-columns. The binding of the antibody to the FLAG peptide was monitored as quenching of the peptide emission.

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References

1. Borman, S. Proteomics: Taking Over Where Genomics Leaves Off. *Chem. & Eng. News* **78**, 31-37 (2000).
2. Rowe, C.A. et al. Array biosensor for simultaneous identification of bacterial ; viral ; and protein analytes. *Analytical Chemistry* **71**, 3846-3852 (1999).
3. Paddle, B.M. Biosensors for chemical and biological agents of defence interest. *Biosensors & Bioelectronics* **11**, 1079-1113 (1996).
4. Wortberg, M., Orban, R.R. & Cammann, K. in Handbook of Biosensor and Electronic Noses: Medicine, Food and the Environment. (ed. E. Kress-Rogers) (CRC Press, Boca Raton FL; 1997).
5. Quake, S.R. & Scherer, A. From micro- to nanofabrication with soft materials. *Science* **290**, 1536-1540 (2000).
6. Purcell, E.M. Life at Low Reynolds Number. *Am. J. Physics* **45**, 3-11 (1977).
7. Jensen, K. Chemical Kinetics: Smaller, faster chemistry. *Nature* **393**, 735-737 (1998).
8. Brody, J.P., Yager, P., Goldstein, R.E. & Austin, R.H. Biotechnology at Low Reynolds Numbers. *Biophys. J.* **71**, 3430-3441 (1996).
9. Knight, J.B., Ashvin, V., Brody, J.P. & Austin, R.H. Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds. *Phys. Rev. Lett.* **80**, 3863-3866 (1998).
10. Chen, C., Wagner, H. & Still, W.C. Fluorescent, Sequence-Selective Peptide Detection by Synthetic Small Molecules. *Science* **279**, 851-853 (1998).
11. Oleschuk, R.D., ShultzLockyear, L.L., Ning, Y.B. & Harrison, D.J. Trapping of bead-based reagents within microfluidic systems: On-chip solid-phase extraction and electrochromatography. *Analytical Chemistry ;* **72**, 585-590 (2000).
12. Ruzicka, J. Lab-on-valve: universal microflow analyzer based on sequential and bead injection. *Analyst ;* **125**, 1053-1060 (2000).
13. Nolan, J., Chambers, J.D. & Sklar, L.A. in Cytometry Approaches to Cellular Analysis. (eds. G. Babcock & R. P.) (Wiley-Liss, 1998).
14. Buranda, T. et al. Ligand Receptor Dynamics at Streptavidin Coated Particle Surfaces: A Flow Cytometric and Spectrofluorimetric Study. *J. Phys. Chem. B.* **103**, 3399-3410 (1999).
15. Nolan, J.P. & Sklar, L.A. The emergence of flow cytometry for sensitive, real-time measurements of molecular interactions. *Nature Biotech.* **16**, 633-638 (1998).
16. Nolan, J.P., Lauer, S., Prossnitz, E.R. & Sklar, L.A. Flow cytometry: A useful tool for all phases of drug discovery. *Drug Discovery Today* **4**, 178-189 (1998).
17. Delamarche, E. et al. Microfluidic Networks for Chemical Patterning of Substrates: Design and Application to Bioassays. *J. Am. Chem. Soc.* **120**, 500-508 (1998).
18. Hanninen, P. et al. A new microvolume technique for bioaffinity assays using two-photo excitation. *Nature Biotech.* **18**, 548-550 (2000).
19. Selvin, P.R. The renaissance of fluorescence resonance energy transfer. *Nature Struct. Biol.* **7**, 730-734 (2000).

20. Chilkoti, A. & Stayton, P.S. Molecular Origins of the Slow Streptavidin-Biotin Dissociation Kinetics. *J. Am. Chem. Soc.* **117**, 10622-10628 (1995).
21. Jung, L.S. et al. Surface plasmon resonance measurement of binding and dissociation of wild-type and mutant streptavidin on mixed biotin-containing alkylthiolate monolayers. *Sens. Actuat. B.* **54** (1999).
22. Buranda, T., Lopez, G.P., Simons, P., Pastuszyn, A. & Sklar, L.A. Real Time Detection of Epitope-Tagged Proteins in Flow Cytometry: FRET Based Assays on Beads with Sub-Picomole Resolution. *Anal. Biochem.* **submitted** (2000).
23. Slootstra, J.W., Kuperus, D., Pluckthun, A. & Meloen, R.H. Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5. *Mol Divers* **2**, 156-164 (1997).
24. Buranda, T., Lopez, G.P., Keij, J., Harris, R. & Sklar, L.A. Peptides, Antibodies, And FRET on Beads in Flow Cytometry: A Model System Using Fluoresceinated and Biotinylated β-Endorphin. *Cytometry* **37**, 21-31 (1999).
25. Gruber, H.J., Marek, M., Schindler, H. & Kaiser, K. Biotin-Fluorophore Conjugates with Poly(ethylene glycol) Spacers Retain Intense Fluorescence after Binding to Avidin and Streptavidin. *Bioconjugate Chem.* **8**, 552-559 (1997).
26. Cafiso, D.S. Interaction of natural and model peptides with membranes. *Current Topics in Membranes* **48**, 197-228 (1999).
27. Brizzard, B.L., Chubet, R.G. & Vizard, D.L. Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* **16**, 730-735 (1994).
28. Knappik, A. & Pluckthun, A. An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. *Biotechniques* **17**, 754-761 (1994).
29. Fung, B.K. & Stryer, L. Surface Density Determination in Membranes by Fluorescence Energy Transfer. *Biochem.* **17**, 5241-5248 (1978).
30. Sklar, L.A., Doody, M.C., Gotto, A.M. & Pownall, H.J. Serum Lipoprotein Structure: Resonance Energy Transfer Localization of Fluorescent Lipid Probes. *Biochemistry* **19**, 1294-1301 (1980).
31. Bourdillon, C., Demaille, C., Moiroux, J. & Saveant, J.-M. Activation and Diffusion in the Kinetics of Adsorption and Molecular Recognition on Surfaces. Enzyme-Amplified Electrochemical Approach to Biorecognition Dynamics Illustrated by the Binding of Antibodies to Immobilized Antigens. *J. Am. Chem. Soc.* **121**, 2401-2408 (1999).
32. Vijayendran, R.A., Ligler, F.S. & Leckband, D.E. A Computational Reaction-Diffusion Model for the Analysis of Transport-Limited Kinetics. *Anal. Chem.* **71**, 5405-5412 (1999).
33. Lagarias, J.C., Reeds, J.A., Wright , M.H. & Wright , P.E. Convergence properties of the Nelder-Mead simplex method in low dimensions. *SIAM Journal on Optimization* **9**, 112-147 (1998).

Figure Captions.

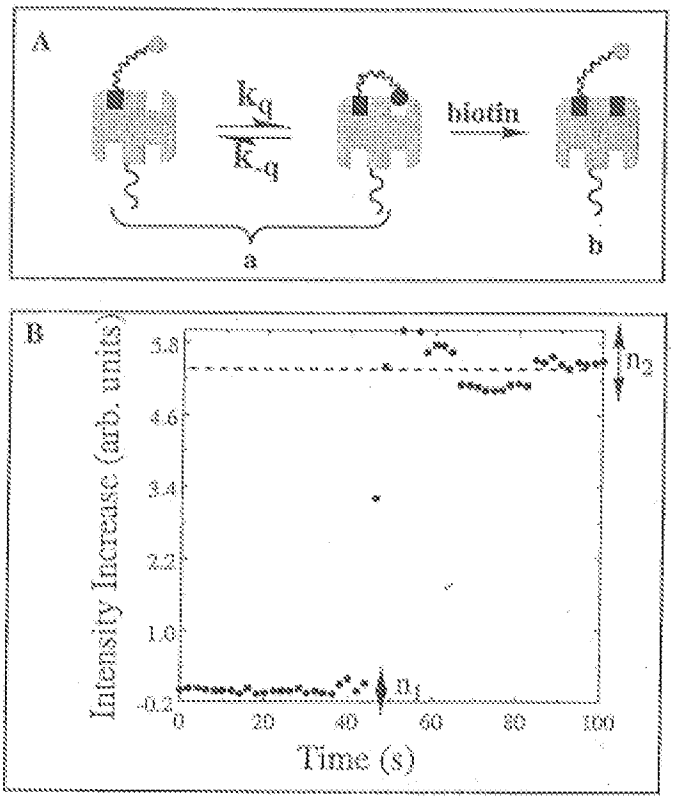
Figure 1. Schematic of a microfluidic apparatus showing the configuration in which sample was delivered and fluorescence measurements taken with a spectrofluorimeter. Elastomeric silicone microchannel, mounted on glass slide with two openings for sample delivery and egress. The micro-channel is 250μm wide, 50μm deep and 3 cm long. Patterned features (inset) 20μm apart, act as filters for holding 30μm borosilicate beads. Thirty thousand, 6.2 μm streptavidin coated beads form a ≈ 600μm long affinity microcolumn.

Figure 2. A. Schematic depiction of: (a) fluorescein biotin bound to streptavidin and subsequent ("ostrich") quenching interaction of fluorescein with a *cis* – binding pocket, on the streptavidin, (b) addition of native biotin blocks ostrich quenching leading to increased emission intensity by fluorophore. **B.** Addition of excess native biotin to beads in channel causes a five-fold increase in fluorescence, corresponding to the process in (b) in the scheme above. The time resolution marks the progress of the fluid through the column of beads. The noise level is indicated by n_1 and n_2 before and after addition of native biotin.

Figure 3. A. FRET transduced passage of a 2μL plug of Texas-Red labeled monoclonal anti-FLAG antibodies (TR-M1) through affinity micro-columns of fluorescein labeled FLAG peptide-bearing beads. The points refer to the normalized intensity readings taken during each run. The lines represent least squares fits to the data (using Equation 2) resulting in the determination of the average association and dissociation rate constants: $k_f = (9.0 \pm 6.0) \times 10^6 M^{-1} s^{-1}$, $k_b = (1.2 \pm 0.8) \times 10^{-3} s^{-1}$. **B.** Sigmoidal dose-response binding curve of TR-M1 mAbs obtained after passage through the affinity microcolumn (inset). Along the y-axis, I_0 is the initial intensity of beads; I_i is the intensity of beads after binding to TR-M1 mAbs, of a given concentration and I_f corresponds to the intensity of beads after binding to the saturating concentration of TR-M1. Data represents three different measurements per concentration of TR-M1. The dissociation constant from the analysis is ≈ 10 nM.

Figure 4. Binding of TR-M1 mAbs to bead-borne FLAG peptides in flow cytometry (K_d ≈ 4.0 nM). Normalized intensities are derived from the means of fluorescence histograms (inset) of bead suspensions incubated with various concentrations of TR-M1 mAbs, and normalized to bead intensity prior to exposure to TR-M1 mAbs.

Figure 5. Model multi-analyte detection array from a segmented affinity micro-column comprised of beads bearing receptors for different analytes (A_i). Each array may be associated with differently tagged receptors to be interrogated at given excitation wavelengths; $\lambda_{ex}(i)$ and $\lambda_{em}(i)$ respectively. This approach can be expandable to include parallel microfluidic networks, with individual sample delivery ports or a single one with several downstream branches.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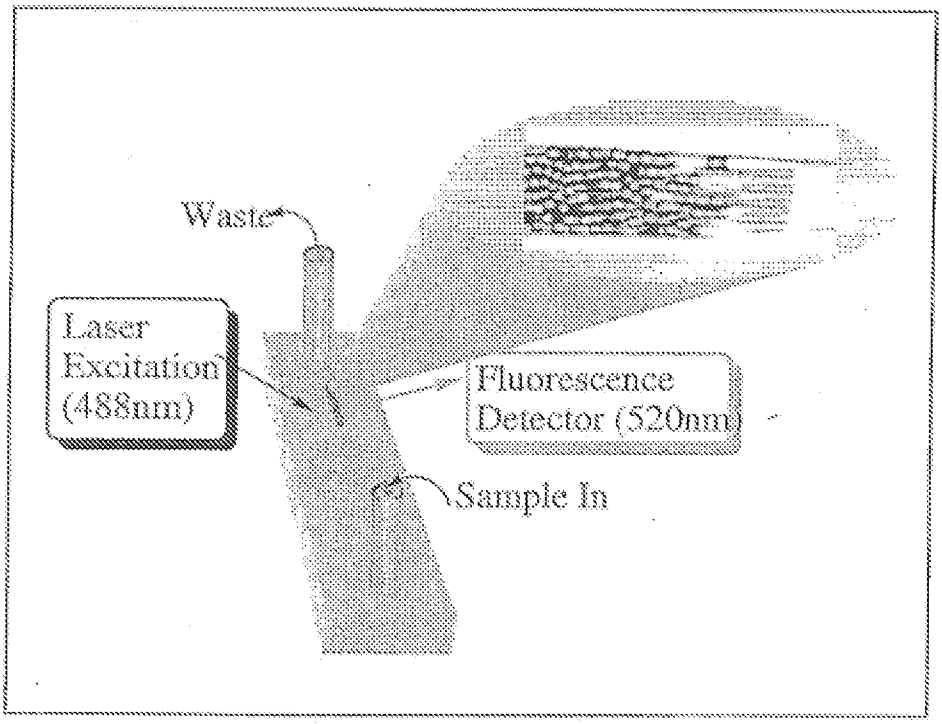
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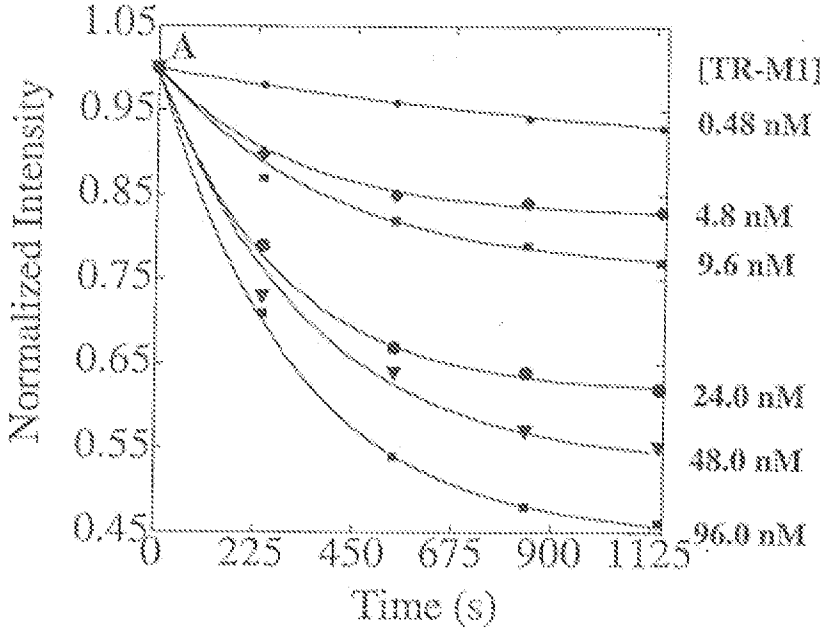
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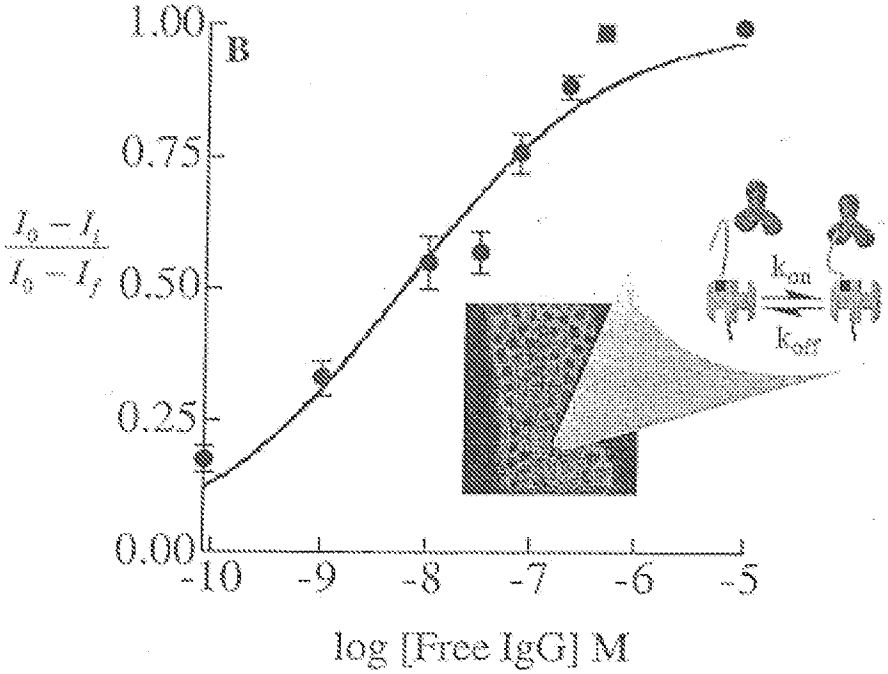
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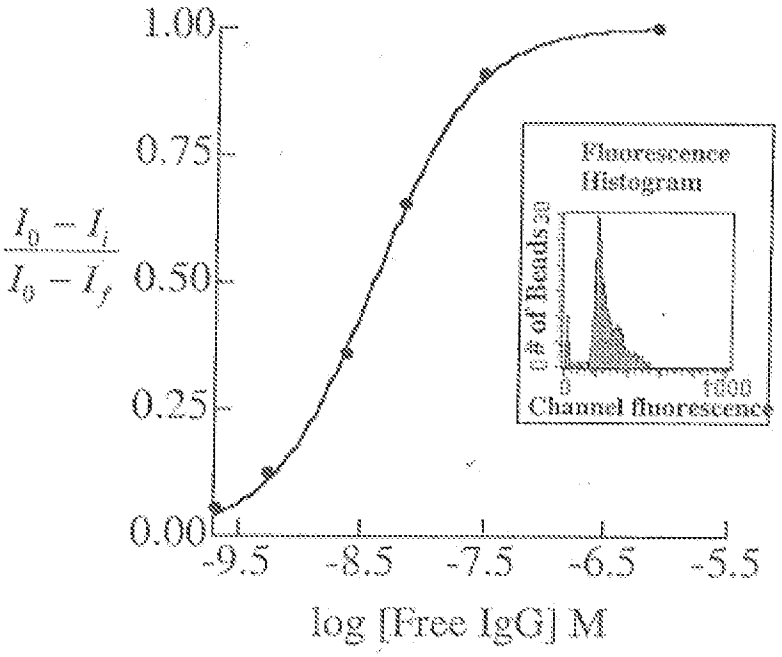
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